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PAPER

## Colonising new frontiers—microarrays reveal biofilm modulating polymers†

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Polymer microarrays provide an innovative approach to identify materials with novel bacterial binding or repellent properties which could subsequently be used in a variety of practical applications. Here, we report a polymer microarray screen of hundreds of synthetic polymers to identify those which either selectively capture the major food-borne pathogen, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*), or prevent its binding. A parallel study with a lab strain of *Escherichia coli* (*E. coli*) is also reported; revealing polymers which either display a common binding activity or which exhibit species discrimination. Moreover, substrates were also uncovered which showed no binding of either organism, even when cultured at high density. The correlation between polymer structure and microbial-modulating behaviour was analysed further, while SEM analysis allowed visualization of the detailed interactions between surface and bacteria. Such polymers offer many new opportunities for bacterial enrichment or surface repulsion, in cleaning materials, as surface coatings for use in the food production industry or as a “bacterial scavenger” resin.

## 1 Introduction

It is well known that bacterial cell surface charge, cell density, and the presence of a variety of microbially produced compounds such as exopolysaccharides are determinant factors in the adhesion process, but other physicochemical features such as pH, temperature, composition of growth media and surface conditioning factors are also known to affect surface attachment.<sup>1</sup> In order to control bacterial attachment, there is a need for materials which result in specific bacterial sequestration or repulsion. These materials when discovered could underpin wide-ranging applications in hygiene and bio-fouling and offering for example a means for the rapid isolation of hospital pathogens, or minimisation of surface contamination through the development of microbe repelling surfaces. They could also provide opportunities for innovative intervention approaches, such as the selective reduction of pathogen loads *via* animal feeds. Other

possible application could be the selective capture of bacteria, spores or viruses on cleaning materials used in clinical, industrial and domestic environments. Minimising attachment and colonisation could be benefit in areas, ranging from artificial implants to packaging for food preparation.

Polymer microarrays have become established as a method to identify polymers that can enrich, manipulate or modulate a variety of adherent or suspended mammalian cell types, including stem cells for regenerative medicine or tissue engineering applications.<sup>2–10</sup> In the present study, we assessed the value of the polymer-based microarray platform to identify novel materials which could be used for the rapid and selective capture of major food-borne pathogens or materials capable of limiting or preventing bacterial adhesion onto surfaces.

For the purposes of this study we focused on the adhesion of the food-borne pathogenic bacterium *Salmonella enterica* serovar Typhimurium (strain SL1344),<sup>11</sup> which is a serious pathogen of clinical and veterinary importance<sup>12</sup> globally and is also a substantial problem in the food industry, and the commensal bacterium *Escherichia coli* (strain W3110).<sup>13</sup>

## 2 Experimental

## 2.1 Chemicals and materials

All chemicals were of analytical grade and used as received without further purification. Silane-prep glass slides, tetracycline, sodium cacodylate trihydrate and all the monomers used were from Sigma-Aldrich. Phosphate buffered saline (PBS) tablet was from Oxoid. GeneFrames were from Thermo Scientific, and 2.5% (w/v) glutaraldehyde and 1% (w/v) osmium tetroxide were from Electron Microscopy Sciences. The

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† Electronic supplementary information (ESI) available: Fig S1 LaVision Bio Analyzer 4F/4S BioTech quantification. Fig. S2 Brightfield and fluorescent microscopy imaging of *S. Typhimurium* binding (Pathfinder™, IMSTAR). Fig. S3 Automated counting of *S. Typhimurium* binding. Table S1 Polyacrylates for *S. Typhimurium* strong/poor binding. Table S2 Polyurethanes showing *S. Typhimurium* binding. Table S3 Polyacrylate series with poor *S. Typhimurium* binding. Fig S4 Fluorescent microscopy image of *S. Typhimurium*-GFP binding on a 5 × 5 spot polymer microarray. See DOI: 10.1039/c0jm01987a

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rectangular four-well plates were from Nunc. Gridded glass coverslips were from CELL-VU.

## 2.2 Polymer microarray fabrication

Polymer microarrays were prepared as previously reported.<sup>7,14</sup>

## 2.3 Culture of bacteria

*S. Typhimurium* and *E. coli* transformed with pHG60 (referred to as *S. Typhimurium*-GFP and *E. coli*-GFP)<sup>15,16</sup> were grown overnight with aeration at 37 °C or 30 °C respectively in Luria-Bertani (LB) broth containing tetracycline (10 µg mL<sup>-1</sup>). Cultures were collected by centrifugation, washed with fresh LB broth and diluted tenfold to a final concentration of approximately (2 × 10<sup>8</sup> CFU mL<sup>-1</sup>) for microarray binding studies.

## 2.4 Bacterial binding

Either *S. Typhimurium*-GFP or *E. coli*-GFP was added to polymer microarrays (in duplicate) in a four-well plate and incubated overnight (except where stated) at room temperature. Subsequently, the polymer microarray slides were washed robustly three times with PBS, rinsed in deionised water, and dried with a stream of air. A GeneFrame and a coverslip (1.9 × 6.0 cm, AB-0630) were then applied to each slide and cleaned with 70% ethanol. Polymer microarrays were analysed using a LaVision BioAnalyzer 4F/4S scanner with a FITC filter. Bacterial adhesion was evaluated *via* integration of the fluorescence intensity after background correction (see ESI, Fig. S1†). The average and standard deviation for sets of four identical polymer features were determined, with the reproducibility between two identical microarrays evaluated by a Student's *t*-test. Polymers with *p*-values <0.001 and 6 degrees of freedom were considered statistically significant.

## 2.5 Fluorescence-based high-content imaging

High-content imaging was carried out using an automated fluorescent microscope with an XYZ stage running Pathfinder™ (IMSTAR) that allowed the capture of single images for each polymer spot. Bacteria were imaged with both brightfield and fluorescein channels with a ×20 objective (see ESI, Fig. S2†).

## 2.6 SEM analysis

Bacteria on the polymer samples were washed (×2) with 0.1 M cacodylate buffer (pH 7.4) and then fixed with 2.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h. Samples were post-fixed with 1% (w/v) osmium tetroxide for 1 h at room temperature, dehydrated stepwise with ethanol (50, 70, 90 and 100% (v/v)), critical point dried in CO<sub>2</sub> and gold coated by sputtering. The samples were examined with a Philips XL30CP Scanning Electron Microscope.

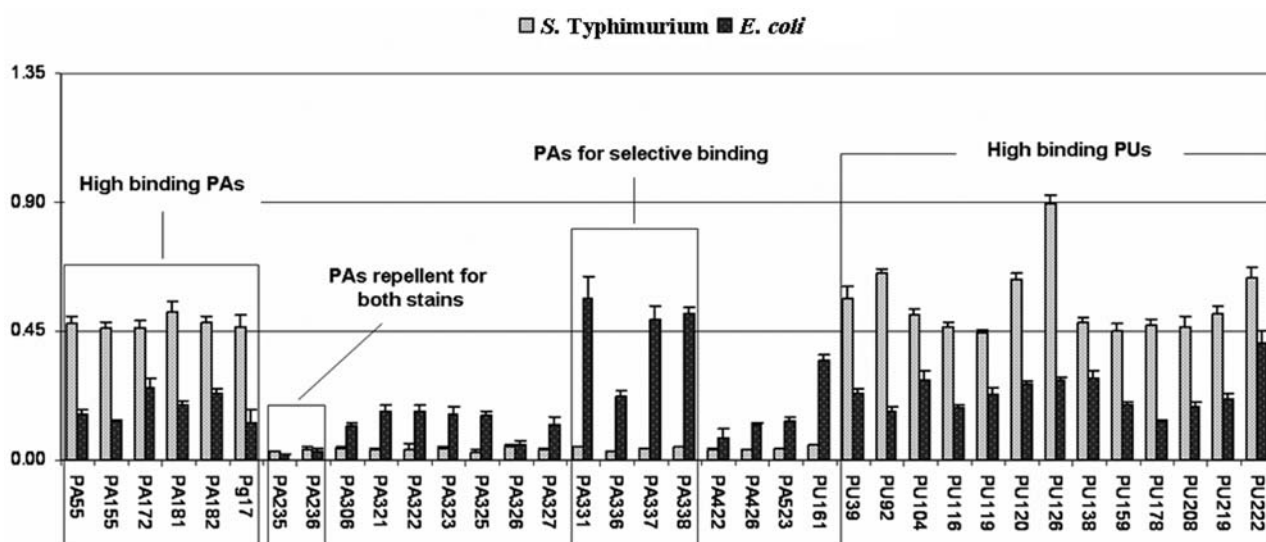
## 2.7 Coverslip scale-up

Polymers were spin-coated onto gridded glass coverslips (DRM 800) and incubated with *S. Typhimurium*-GFP and imaged *via* SEM. The numbers of bacteria in randomly selected sub-squares (four for each coverslip) were counted with Image-Pro Plus 4.5 (©2001 Media Cybernetics) (see ESI, Fig. S3†).<sup>17</sup> Reproducibility was determined by calculating the average and the standard deviation for the four identical sub-squares.

## 3 Results and discussion

### 3.1 Analysis of bacteria attachment

Analysis was enabled by the expression of Green Fluorescent Protein (GFP)<sup>15</sup> within the bacteria, allowing detection of bacterial binding on a polymer microarray of 370 polyurethanes (PUs) and polyacrylates (PAs).<sup>14</sup>



**Fig. 1** Analysis of *S. Typhimurium* and *E. coli* binding on the polymer microarrays. PA and PU library members showing strong/poor *S. Typhimurium* and/or *E. coli* binding. Binding is expressed as background corrected mean fluorescent intensity with error bars representing the standard deviation. X-axis: polymer code. Y-axis: fluorescent intensity in arbitrary units (au).

Analysis revealed six PAs and thirteen PUs which showed strong binding of *S. Typhimurium* (Fig. 1). *E. coli* affinity was weaker in general, but varied with the particular polymer.

Four of the six high binding PAs (155, 172, 181 and 182) (Fig. 1) contained the monomer 2-hydroxyethylmethacrylate

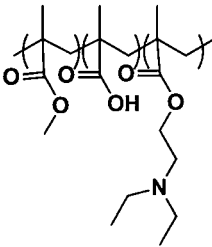
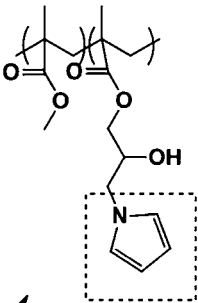
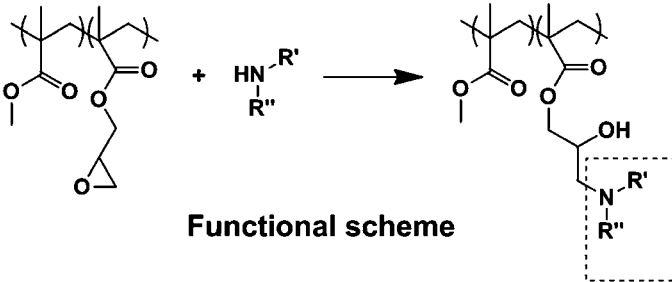
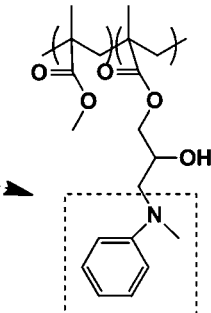
(HEMA) (see ESI, Table S1†) and of those four, two (PA181 and 182) contained the monomer 1-vinylimidazo (VI) within monomer ratio: 70/30 and 50/50, respectively (Table 1).

On the other hand, polymer structure analysis of the PUs revealed that the diols polybutylene glycol (PTMG) and

**Table 1** Structure of the strong binding PAs and PUs

<b>PA181/182</b>		<b>Molecular ratio:</b> HEMA/VI 70/30 (PA181) 50/50 (PA182)
<b>PU178</b>		
<b>PU222</b>		
	$R = -CH_2(CH_3)_4CH_2-$ $-CH_2C(CH_3)_2CH_2-$	<b>Molecular ratio:</b> PU178: PTMG/HDI/NMPD (25/52/23) PU222: PHNAD/BICH/OFHD (25/52/23)

**Table 2** Scheme of the poor binding polymer functionalisation and structure of the selected poor binding PAs

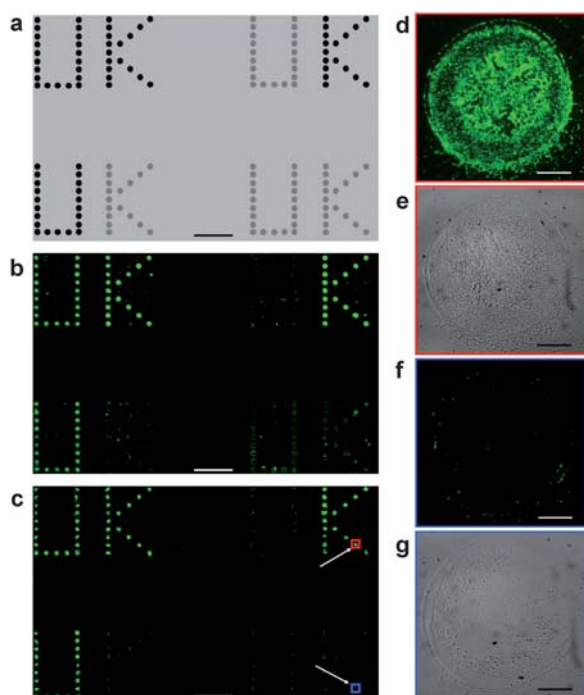
	<p>Molecular ratio: MMA/MA-H/DEAEMA 70/15/15 (PA235) 70/10/20 (PA236)</p>	<p><b>PA235/236</b></p>		<p>Molecular ratio: MMA/GMA 70/30 (PA331) Functional group: Pyrrole</p>	<p><b>PA331</b></p>
	<p><b>Functional scheme</b></p>	<p><b>Functional group</b></p>		<p>Molecular ratio: MMA/GMA 90/10 (PA336) 70/30 (PA337) 50/50 (PA338) Functional group: MAn</p>	<p><b>PA336/337/338</b></p>



polypropylene glycol (PPG) were common in ten of the thirteen “hit” polymers (see ESI, Table S2†). Thus, for example, polymer PU222 showed good-binding of *S. Typhimurium* and *E. coli*, whereas there was a substantial difference between the binding of *S. Typhimurium* and *E. coli* on polymer PU178 (Table 1).

Sixteen PAs showed substantial inhibition of *S. Typhimurium* adhesion (Fig. 1), with thirteen containing the monomer methyl methacrylate (MMA), and with eleven of these also containing the derivatisable monomer glycidyl methacrylate (GMA) (Table 2 (functional scheme) and ESI: Tables S1† (poor binding polymers) and S3† (different functionalisations)).

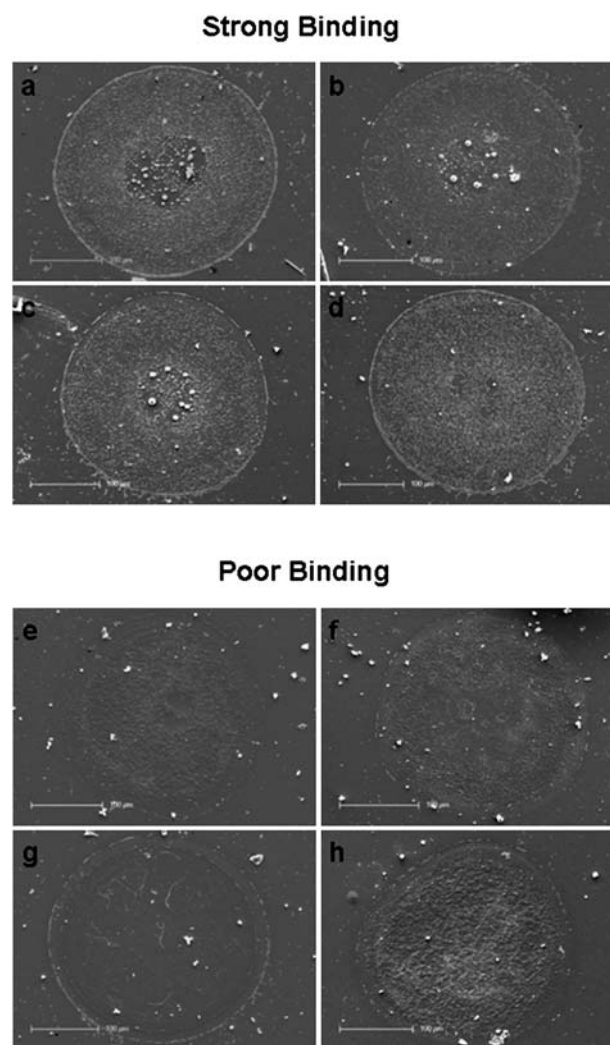
PA235 and PA236, which were composed of methyl methacrylate (MMA), methacrylic acid (MA-H) and 2-(diethylamino)ethyl methacrylate (DEAEMA), were highly successful in preventing adhesion of both *S. Typhimurium* and *E. coli* (Table 2). Polymers PA331, PA337 and PA338 selectively bound *E. coli*, but did not bind *S. Typhimurium*, with PA337 and PA338 differing only in the molar ratios of the relevant monomers (MMA and GMA): 70/30 (PA337) and 50/50 (PA338) (Table 2). The related polymer PA336 (90/10) showed a similar trend, but with slightly less selectivity (Table 2). This suggested the importance of GMA functionalisation with *N*-methylaniline (MAN) in making this group of polymers selective for *E. coli* (see also ESI, Table S3†).



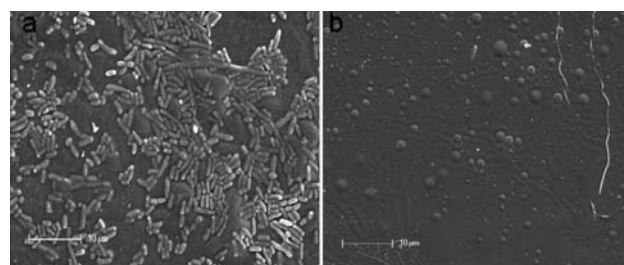
**Fig. 2** *S. Typhimurium* attachment/repulsion: (a) array design with the binding polymer PU104 (in black) and the poor binding polymer PA325 (in grey); (b) BioAnalyzer scanning of the array using a fluorescein filter; (c) fluorescent microscopy imaging ( $\times 20$  objective). Scale bar = 4 mm. Arrows indicate fluorescent and brightfield microscopy images of *S. Typhimurium* grown on representative polymer spots: (d) fluorescein channel and (e) brightfield of PU104; (f) fluorescein channel and (g) brightfield of PA325. Scale bar = 100  $\mu\text{m}$ .

### 3.2 Reproducibility

Following the initial analysis of the entire library (in duplicate and with eight copies of each polymer), several polymers which resulted in the strongest or poorest binding of *S. Typhimurium* were re-printed and re-examined with each polymer printed in



**Fig. 3** SEM images of *S. Typhimurium* strong/poor binding on selected polymer spots. Strong binding: (a) PU104; (b) PU126; (c) PU120; and (d) PA155. Poor binding: (e) PA426; (f) PA422; (g) PA325; and (h) PA235. Scale bar = 100  $\mu\text{m}$ .



**Fig. 4** High magnification SEM images of *S. Typhimurium* binding/non-binding on selected polymers: (a) PA155 and (b) PA325. Scale bar = 10  $\mu\text{m}$ .

a  $5 \times 5$  pattern. Of the four good polymers examined (PU104, PA155, PU120, and PU126), each showed consistent cellular attachment, whilst the four poor binding polymers (PA325, PA422, PA426, and PA235) confirmed their “anti-bacterial” binding properties (see ESI, Fig. S4†).

### 3.3 Impact of time on attachment

It would clearly be advantageous for a polymer to be able to bind bacteria in a rapid time frame. Therefore, to test the rapidity of *S. Typhimurium* binding, an array with the letters ‘UK’ was fabricated using high and low binding polymers (PU104 and PA325, respectively), and *S. Typhimurium* incubated on the array for four hours.

As can be seen in Fig. 2, a uniform binding pattern was observed with PU104, with little binding observed on polymer PA325.

*S. Typhimurium* binding on several selected polymers which resulted (PU104, PA155, PU120, PU126, PA325, PA422, PA426 and PA235) was assessed with particular attention paid to the binding characteristics and polymer spot morphology (Fig. 3).

Bacteria appeared firmly attached and closely packed on PA155, aligning along their longitudinal axis. Small micro-colonies were observed on the strong-binding polymer surface (Fig. 4a). In contrast, non-binding polymers (PA325) showed little attachment and no evidence for early biofilm formation, implicating these polymers as potential new materials for anti-bacterial surface coatings (Fig. 4b).

### 3.4 Scale-up analysis

In order to see if the selected polymers could be scaled-up and, to find whether those polymers could be used in practical applications, PA155 and PA325 were spin-coated onto glass coverslips,

which were formed of a central square ( $1 \times 1$  mm) subdivided in one hundred squares ( $100 \times 100 \mu\text{m}$ ). These coated coverslips, and uncoated coverslips (as a control), were incubated with *S. Typhimurium*-GFP as previously reported (Section 3.1) and imaged *via* SEM (Fig. 5a–c). The number of bacteria on randomly selected subsquares on the coverslips were counted to give the number of bacteria per  $\text{mm}^2$  (Fig. 5d). The analysis of binding on both coated and uncoated coverslips confirmed the expected results. *S. Typhimurium* attached onto polymer PA155 with a 7-fold increase in binding compared to an uncoated coverslip, whereas the number of *S. Typhimurium* on the anti-binding polymer PA325 was twenty times less than the glass control (Fig. 5d).

## 4 Conclusion

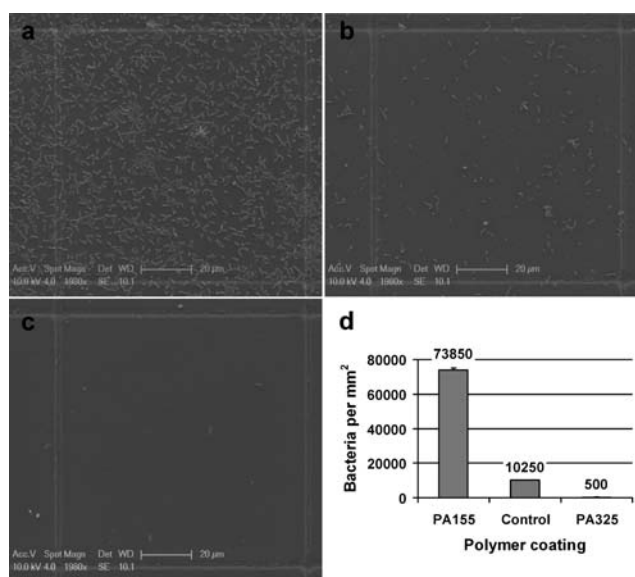
Polymer microarrays were successfully used for the identification of polymers which bound either *S. Typhimurium* and/or *E. coli* or prevented their colonisation of surfaces, with fluorescence imaging that allowed the rapid, parallel, and comprehensive, evaluation of bacterial adhesion on 370 polymers. Binding and non-binding surfaces were shown to be highly dependent on both the chemical structures and properties of the polymers, and were sufficient to allow discrimination between adhesive properties of different bacterial genera. For the strongest binding polymers SEM revealed the formation of early biofilm-like micro-colonies, where cells were longitudinally aligned and closely packed, whereas a number of polymers were also identified which clearly prevented bacterial attachment, even at very high cell densities. Identified polymers are now being developed as coating materials to help reduce hospital endotracheal tube infections as well as in *Campylobacter jejuni* and *Clostridium difficile* infections.

## Acknowledgements

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**Fig. 5** SEM images and analysis of the *S. Typhimurium* binding on the selected polymer coated coverslips: (a) PA155 (strong binding); (b) control (no-polymer coated) and (c) PA325 (non-binding). Scale bar: 20  $\mu\text{m}$ . (d) The average number of bacteria (*S. Typhimurium*) per  $\text{mm}^2$  on PA155 (strong binding) and PA325 (non-binding) coated coverslips ( $n = 4$ ).

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